Direct Reprogramming of Human Cell Lines *in vitro* Towards a β-Cell Phenotype

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Abstract

Type 1 diabetes results from the loss of insulin producing β-cells of the pancreas, causing dependence on exogenous insulin for those affected. β-cell transplantation has been a promising treatment method, but lack of donor cells and requirement for repeated treatments currently limit this approach. Alternative treatments for type 1 diabetes are investigating reprogramming cell types toward a β-cell-like phenotype to generate a reliable source of insulin-secreting cells for transplant therapies. The pancreatic transcription factors Pdx1, Ngn3, and MafA have been combined in an adenoviral vector (Ad-PNMa) and used to reprogram rodent cells to generate insulin-secreting cells both *in vivo* and *in vitro*. However, studies examining similar reprogramming of human cell lines have met with little success to date. Here, we attempt to reprogram a human hepatocarcinoma cell line with the Ad-PNMa vector, and human pancreatic epithelial ductal (HPDE) cell lines with the Ad-PNMa vector and new Ad-PN and Ad-PNMb vectors expressing human Pdx1, Ngn3, and MafB. It was found that qRT-PCR detected weak insulin gene expression in Ad-PNMa infected HepG2 cells. Infection of HPDE cells with Ad-PNMb vector caused approximately 64% of cells to express the three vector cargo genes when treated at a concentration of $1.7x10^{10}$ ifu/ml. No insulin production was detected in HDPE cells following vector treatments, indicating that expression of these genes is not sufficient to induce insulin expression in HPDE cells. It may be possible to reprogram the HPDE cells to a β-cell phenotype with the expression of additional genes.

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1 | Introduction

1.1 | Scope and Definition of Diabetes

Diabetes is a common medical condition throughout the world. Developed countries have seen an increase in the incidence of this condition in the past several decades, largely due to shifts toward more sedentary lifestyles and changes in the composition of typical diets (GDB 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016; NCD Risk Factor Collaboration, 2016). With increasing numbers of people affected in recent times, the need for advancements in the treatment and understanding of this disease has become ever more apparent. Global prevalence rates have increased from 4.3% to 9.0% in men and 5.0% to 7.9% in women between 1980 and 2014 (NCD Risk Factor Collaboration, 2016). The number of people living with diabetes increased from 108 million to 422 million in the same time frame. These cases are divided between three forms: type 1, type 2, and gestational diabetes. Each type of diabetes is associated with its own set of causes and the degree to which it may be treated, with type 1 and type 2 being the most problematic.

1.2 | Type 2 Diabetes

Type 2 diabetes, also referred to as adult-onset diabetes, is the most common form of the condition, affecting approximately 90% of those diagnosed with the disease. In total, type 2 diabetes results in estimated costs over $825 billion worldwide from treatment and diagnosis (NCD-RisC, 2016). Type 2 diabetes is currently the sixth leading cause of physical disability in the world, placing strong socioeconomic costs on those afflicted (GDB, 2016). The cause of this form of diabetes is a dysfunction in the body's response to insulin, referred to as insulin resistance. In this condition, cells that would normally respond to insulin and remove glucose

from the blood fail to do so, resulting in hyperglycemia in the majority of patients. Obesity, lack of exercise, and poor diet place individuals at high risk for this condition, though some genetic factors have been found to also play a role (Reviewed in Atkinson et al., 2014). Standard complications associated with unresolved type 2 diabetes include neuropathy, cardiovascular disease, and decreased blood flow that can result in amputation of affected limbs (UKPDS, 1998). Depending on the severity of the condition and the length for which it has persisted, treatment can be accomplished with simple lifestyle and diet changes (Davies et al., 2015; Steven et al., 2016). Pharmaceutical interventions remain an option for some cases, with multiple drugs being readily available to enhance glucose uptake from the bloodstream or to cause resensitization to insulin. Many of the drugs involved in these therapies have the potential to cause serious side effects and there is no clear-cut rule on which compounds should be tried first and in what combinations (Davies et al., 2015; Kahn et al., 2011). Regardless of the treatment path pursued, blood glucose monitoring and attempts to control glucose intake are important for minimizing the risks associated with type 2 diabetes (UKPDS, 1998).

1.3 | Type 1 Diabetes

The onset of type 1 diabetes typically occurs in childhood is the result of the body's inability to produce insulin (Reviewed in Daneman, 2006). This form of diabetes is associated with destruction of the insulin-producing β-cells of the pancreas due to an auto-immune response, and not from β-cell dysfunction or a failure of other tissues to respond to insulin. Causes for this condition have been widely studied but remain poorly understood, and are largely attributed to specific genetic or environmental factors (Lambert et al., 2004; Narayan et al., 2003). Since they lack the ability to produce insulin, type 1 diabetic patients must rely on exogenous insulin supplements. Typically, these supplements are delivered through injection or

by pumps that patients carry on their person at all, or most, times of the day. Complications include infections caused by difficulties in either applying or maintaining insulin delivery equipment and pathologies caused by the disease, such as cardiovascular disease, stroke, and other conditions also associated with type 2 diabetes (Reviewed in Daneman, 2006; DCCT Research Group, 1993).

Attempts to cure type 1 diabetes have relied on allogenic transplantation of islets, the clusters of β-cells found in the pancreas, or through whole pancreas transplantation. These methods have a particular weakness in that appropriately matched donors may not be readily available (Landsberg and Shapiro, 2010). Transplantation methods were traditionally difficult due to risks of rejection, though the development of the Edmonton Protocol significantly alleviated this issue in β-cell transplants. In coupling the transplant process with the application of glucocorticoid-free immunosuppressants, greater success has been achieved (Shapiro et al., 2000). These methods do not always result in permanent independence from a need for exogenous insulin supplementation. At the 3 year mark following transplant at the time of the Edmonton protocol's development, only 27% of patients retained insulin independence (Barton et al., 2012), and this number fell to only 10% after the 5 year mark (Ryan et al., 2005). Improvements in methods have allowed the 3 year insulin independence percentage to increase to 44% following transplantation in the 2007-2010 period (Barton et al., 2012), with continued success allowing insulin independence at 5 years to reach 58-60% (Lablanche et al., 2015; Qi et al., 2014). However, although the increasing success of these methods to provide long-term insulin independence is encouraging, it is apparent that more research must be done to improve the availability and sustainability of islet transplantation or provide new sources of β-cells. As such, type 1 diabetes continues to pose a significant threat to the well-being of affected

individuals, and lacks the high number of relatively simple treatment possibilities present with either gestational or type 2 diabetes.

1.4 | Gestational Diabetes

Gestational diabetes refers to insulin resistance or intolerance conferred to the mother during pregnancy. Only about 7% of pregnancies result in this condition, though risk increases depending on the circumstances of the pregnancy and whether there are concurrent conditions such as obesity. Management is completed with some diet restriction or insulin therapy, though any treatment option must be checked to ensure health of the developing fetus (American Diabetes Association, 2004). This condition typically does not continue past completion of pregnancy and is associated with few long-term consequences for the mother, the most significant of which being an increase in the chance of developing type 2 diabetes, however there is some heightened risk of miscarriage. Children resulting from these pregnancies are also at increased risk for developing type 2 diabetes and obesity (American Diabetes Association, 2004; Kim et al., 2010).

1.5 | Surgical Diabetes

Surgical diabetes results when the pancreas is surgically removed, either due to organ failure, cancer, or chronic pancreatitis, and is often the last step in management of some conditions (Reviewed in Matsumoto, 2011). In some cases, the β-cell islets are removed from the pancreas once it has been removed and are then re-implanted into the patient to prevent diabetic complications. This procedure of total pancreatectomy and islet auto-transplant (TPIAT) typically has good outcomes because the cells are not rejected by the patient, auto-immune suppressants are not required, cells are not stored outside of the body for long periods of time,

and the pancreas has not been subjected to a cytokine storm that can occur in pancreases from deceased donors (Reviewed in Matsumoto, 2011).

1.6 | Pancreatic Development

Developing novel treatments for type 1 diabetes requires knowledge of pancreas function and development to permit a closer examination of potential therapies and risks. The pancreas is an organ with three distinct populations of cells: exocrine cells that secrete enzymes, ductal cells that transport exocrine products to the digestive tract, and endocrine cells that secrete hormones. Several populations of cells appear during development of the endocrine system: β-cells responsible for insulin secretion; α-cells responsible for glucagon secretion; δ-cells that secrete somatostatin, a hormone that inhibits secretion of glucagon and insulin; and PP cells that secrete pancreatic polypeptide, a hormone associated with self-regulation of the overall organ (Reviewed in Slack, 1995).

As the primary cell type involved with diabetes, development and fate specification of β-cells is an important area of focus for type 1 diabetes research. Expression of the transcription factor Pdx1 is necessary for initial development of the pancreas, with upregulation of Pdx1 expression being required for the production of all three pancreatic tissue types (Figure 1) (Gu et al., 2002; Stoffers et al., 1997). Other key transcription factors involved in early pancreatic organogenesis include Hlxb9, Hnf6, Ptf1a, and Nkx6.1 (Reviewed in Wilson et al., 2003). Upregulation of Pdx1 protein expression in the adult pancreas is required for the expression of various β-cell genes, including insulin. In animal models, when Pdx1 is repressed in the adult pancreas, β-cells regress from their specified phenotype and are no longer capable of producing insulin, eventually leading to a diabetic state (Ahlgren et al., 1998). Interestingly, β-cells shift toward an α-cell phenotype upon becoming Pdx1-negative (Gao et al., 2015), whereas concurrent

exogenous expression of Pdx1 with MafA, a late-stage transcription factor, in α-cells has been shown to induce insulin expression in these cells (Xiao et al., 2018), suggesting that Pdx1 may be involved with the downregulation of alpha cell-specific factors.

Following initial pancreatic organogenesis, the next critical cell fate specification step is determination of endocrine and exocrine cell populations. The transcription factor Neurogenin 3 (Ngn3) is crucial for induction of endocrine cell fate in cells also positive for Pdx1 (Apelqvist et al., 1999; Sommer et al., 1996). When delivered exogenously to adult pancreatic ductal cells, Ngn3 has been shown to induce differentiation toward endocrine character and increase the population of insulin-positive cells, highlighting its connection to β-cell specification (Heremans et al., 2002).

In the pancreas of mice, the factor MafA is exclusively expressed in β-cells and appears to have the unique ability to induce the expression of insulin in the adult β-cell (Artner et al., 2008). Past studies have revealed 3 enhancer binding sites responsible for the expression of the insulin gene, one of which is bound by MafA, while the other two are bound by Pdx1 and NeuroD1 (Olbrot et al., 2017). The transcription factor MafB is closely related to MafA and appears to play a key role in the maturation of both β-cells and α-cells. Elimination of the MafB gene in embryonic development results in a reduction in the number of both insulin- and glucagon-positive cells (Artner et al., 2007). However, unlike MafA, the MafB transcription factor retains expression only in α-cells of the adult pancreas where it stimulates the production of glucagon (Artner et al., 2006). Loss of MafB expression gradually occurs as MafA is beginning to be expressed in the differentiating β-cell, suggesting β-cells go through a $MafB^{+}$ $MafA^{-}$ $Insulin^{+}$ step before final maturity (Artner et al., 2010; Nishimura et al., 2006). In contrast, human β-cells go through a different maturation process than mouse β-cells, expressing

MafB both during the initial β-cell specification process and into maturity (Figure 2) (Conrad et al., 2015; Dai et al., 2012). When mature, human β-cells require expression of both MafA and MafB proteins in order to express insulin.

1.7 | Reprogramming Techniques

Understanding β-cell development may enable the possibility of using cellular reprogramming techniques as meaningful methods for developing novel type 1 diabetes treatments. Cellular reprogramming, the process by which one cell type is transformed into another, has become a highly competitive research avenue in recent years. A notable example is the recent use of dopaminergic neurons derived from induced pluripotent stem cells (iPSC) to successfully treat Parkinson's disease in primates, illustrating the importance this has for human medicine (Kikuchi et al., 2017). A key advancement in this field of research was the landmark experiments by Shinya Yamanaka and colleagues, which showed that cells could be forced into an induced pluripotent state *in vitro* following overexpression of the transcription factors Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka, 2006). Many other methods for cellular reprogramming have since been introduced to replace integrating viral delivery of transcription factors. Examples include the use of small molecules for high-efficiency neural induction in human embryonic stem cells (Chambers et al., 2009); epigenetic modifications to make DNA more accessible for neural conversion in *Caenorhabditis elegans* (Patel et al., 2009; Tursun et al., 2011); and miRNA to convert mouse cardiac fibroblasts to cardiomyocytes (Jayawardena et al., 2013). Additional developments of reprogramming techniques continue to refine the field overall and bring research closer to establishing medical treatments for type 1 diabetes and other conditions.

1.8 | Reprogramming Cells to a β-Cell Phenotype in Mouse Models

There has been major interest in reprogramming strategies for the treatment of type 1 diabetes, as this technology creates the possibility of creating endless banks of β-cells for transplant procedures, eliminating the need for donors (Reviewed in Atkinson and Eisenbarth, 2001). Reprogramming to a β-cell-like state may circumvent some of the issues associated with the transplant process, such as immunosuppression, risk of rejection, and the difficulty of finding donor cells. Many reprogramming experiments have focused on mouse models to elucidate the necessary genetic triggers for conversion to a β-cell phenotype (Ariyachet et al., 2016; Banga et al., 2012, 2014; Wang et al., 2016; Zhou et al., 2008). Current reprogramming efforts have included the overexpression of three pancreatic transcription factors previously discussed: Pdx1, Ngn3, and MafA (PNMa) (Zhou et al., 2008). Choice of these three transcription factors for overexpression was based on their association with β-cell determination, and has been established as a working method for reprogramming to a β-cell-like state in mice. The first use of this three-gene combination using adenoviral vectors established that adult pancreatic exocrine cells could be reprogrammed to β-cells *in vivo*, with the reprogrammed cells being described as nearly identical to native β-cells in structure and ability to relieve hyperglycemia (Zhou et al., 2008).

Subsequent experiments have focused on using other cell types to achieve reprogramming to a β-cell-like state using the same gene combination. Experiments using mouse gallbladder have seen limited success, with *in vitro* reprogramming of gallbladder cells resulting in 25% of treated cells reprogramming towards an insulin-positive state following treatment with the transcription factors (Wang et al., 2016). Transplantation of these cells was attempted to provide evidence that such a technique can produce a glucose-sensitive β-cell-like product

capable of surviving in transplant recipients. However, this success is superficial and promise is reduced when looking at the specific characters of these reprogrammed gallbladder cells. These cells retained high gallbladder-specific gene expression and lacked significant overall expression of β-cell genes, there was a failure to alleviate hyperglycemia in the transplant recipients, and a large portion of transplanted cells died despite mice being immunocompromised.

Other experiments have focused on using mouse intestinal organoids, miniature organ systems developed under cell culture conditions, to generate transplantable β-cells (Ariyachet et al., 2016). Cells within intestinal organoids treated with the PNMa three-gene combination were shown capable of being reprogrammed to form insulin-secreting, glucose-sensitive cells that may ameliorate hyperglycemia long-term in transplant recipients. Moreover, the use of organoids produces a unique transplant system in which clustered, mini-organs may be transferred, which may provide higher stability than treatment with suspensions of single cells.

1.9 | Reprogramming Human Cells to a β-Cell Phenotype

Pilot studies with reprogramming human cells towards a β-cell fate have seen limited success. Both embryonic stem cells and iPSC have been used for this differentiation research, with differentiation towards a β-cell-like phenotype being accomplished through treatment with growth factors (D'Amour et al., 2006). Progression through individual developmental stages is achieved by the application of growth factors that trigger the expression of different genes in a manner that mimics the natural progression of β-cell development. Similar methods have shown that differentiated cells can be generated that secrete insulin according to the presence of glucose and form islet-like clusters comparable to islets in the adult pancreas (Kim et al., 2016; Zhu et al., 2016). Akin to the previous mouse experiments, limited studies on the efficacy of the three-gene combination of Pdx1, Ngn3, and MafA have been conducted using human cells,

specifically pancreatic ductal cells (Lee et al., 2013a). These experiments have been shown to produce insulin secreting β-cell-like phenotypes capable of secreting insulin in response to glucose. These findings are preliminary, as transplantation of these reprogrammed cells to an *in vivo* mouse model were unsuccessful, including failure of reprogrammed cells to relieve hyperglycemia in mice past two weeks post-surgery (Kim et al., 2016). Reprogrammed cells have also failed to reproduce all characteristics of native β-cells and a lack in the production of all cell types associated with islets, especially support cells (Zhu et al., 2016). Conversion of human ductal cells to β-cells using Pdx1, MafA, and Ngn3 has similar issues to mouse studies in that reprogrammed cells have significantly lower insulin expression than pancreatic β-cells and transplanted cells are not viable (Lee et al., 2013a).

While these studies provide information on the progress of research into reprogramming human cells toward a β-cell phenotype, much remains to be answered regarding the viability of this approach in future treatment efforts. Even with the limited successes of these reprogramming techniques, each example described contain significant deficiencies, such as poor insulin production or inability to sense glucose levels, that currently prevent it from moving forward as a potential treatment for type 1 diabetes.

1.10 | Past Studies of the Dutton Lab

Focusing on the three transcription factors Pdx1, Ngn3, and MafA, an adenoviral vector has been constructed to deliver and co-express the three genes (hereafter referred to as Ad-PNMa) in cells for reprogramming experiments (Figure 3). Ad-PNMa has been successfully used to reprogram liver cells *in vivo* in diabetic mice to an insulin-producing state (Banga et al., 2012). Following tail vein injection delivery of the Ad-PNMa vector, these treated diabetic mice restored normal blood-glucose levels for approximately 4 months, with insulin-secreting cells

being formed in the liver that were shown to secrete insulin in a glucose-sensitive manner. Using an immunosuppressant in tandem with Ad-PNMa infusion was also successful in alleviating hyperglycemia in wild type diabetic mice, though this required transient additional treatment of the mice with the peroxisome proliferator activated receptor (PPAR) α and γ agonist WY14643 at the time of vector delivery (Banga et al., 2014). The combination of these treatments allowed Ad-PNMa to be applied at a low dose and induce long-term insulin expression that was associated with the formation of Sox9-positive ductal structures. While this is encouraging, the benefits of the reduced Ad-PNMa dose and increased stability of insulin secreting structure must be weighed against the risk of using the PPAR agonist WY14643, which may induce liver hyperplasia (Cohen and Grasso, 1981; Devchand et al., 1996; Ledwith et al., 1996).

1.11 | Goal of the Current Study

This study uses Ad-PNM vectors expressing human coding sequences of the PNM genes to examine the potential of reprogramming human cells toward a β-cell phenotype. However, unlike mouse studies, multiple Ad-PNM vectors have been generated to assess different gene combinations for the activation of human insulin expression in reprogrammed cells. Here, a vector lacking the MafA gene (Ad-PN) and a vector utilizing MafB in place of MafA (Ad-PNMb) is used to test reprogramming of human pancreatic and liver cell lines toward a β-cell phenotype. The efficacy of these vectors in reprogramming is used to determine whether these genes are sufficient to induce insulin expression in human cells.

Should successful reprogramming be shown, additional experiments may involve transplantations in model animals to assess how these reprogrammed cells behave *in vivo* and how competent they are at alleviating hyperglycemic conditions. The experiments will provide

insight into future directions and may develop a novel method of establishing a new source of β-cells for potential use in clinical transplantation procedures.

2 | Methods

2.1 | Cell Culture

A human HepG2 liver cell line originally derived from a 15 year-old male with hepatocarcinoma was used for assays using Ad-PNMa (Knowles and Aden, 1983), and a human pancreatic ductal epithelial (HPDE) cell line was used for assays using Ad-PNMa and Ad-PNMb (Furukawa et al., 1996; Ouyang et al., 2000).

HepG2 cells were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v fetal bovine serum, 1% v/v non-essential amino acids, and 1% v/v antibiotic antimycotic. Passaging was performed when cells reached 100% confluency using 0.25% trypsin.

HPDE cells were cultured in Keratinocyte Serum-Free Media (KSFM) supplemented with epidermal growth factor and bovine pituitary extract. Passaging was performed at 100% confluency with 0.25% trypsin, which was neutralized using defined trypsin inhibitor from soybean.

2.2 | Testing Ad-PNMa, Ad-PN, and Ad-PNMb Vectors

To determine the ability of the HepG2 and HPDE cell lines to be reprogrammed and an optimal concentration of vector to use, functional and dilution assays were performed using Ad-PNMa, Ad-PN, and Ad-PNMb. HepG2 and HPDE cells were passaged into twelve-well tissue culture plates at 50,000 cells per well to assess production of vector proteins using

immunohistochemistry. Six-well plates with 100,000 cells per well were used for RNA isolation. For each Ad-PNMa, Ad-PN, and Ad-PNMb, dilution assays using constant relative volumes of diluted virus were performed. The volumes were 50 µl/50,000 cells, 100 µl/50,000 cells, 200 µl/50,000 cells, and 400 µl/50,000 cells. Original vector titers (Table 1) were diluted 1:1000 in respective culture media prior to infection.

Two-time lengths were selected to determine the optimal amount of time needed for HepG2 cells to reprogram. HepG2 cells were treated with A12 Ad-PNM vector for either 72 or 120 hours. Infections lasting 72-hours were treated with Ad-PNM infected media for 72 hours and then new culture media was applied for 48 hours. Infections lasting 120 hours were treated with Ad-PNM infected media for 120 hours and were not refreshed with new media.

HPDE cells were treated with C13 Ad-PNMa, Ad-PN, or Ad-PNMb vectors for 24 hours, after which new culture media was applied and the cells were left for 72 hours. A negative control featuring no addition of vector was used for each cell plate. Three replicates for each vector were performed.

2.3 | Immunohistochemistry and Cell Counting

To fix the HepG2 and HPDE cell twelve-well plates, 10% formalin (Protocol, Thermo Scientific) was applied for 10 minutes. Plates were treated with antibodies for one of the three pancreatic transcription factors delivered by the viral vector and insulin for each experiment tested. Cells permeablized for 10 minutes using a solution of PBS-T 1% BSA 1% Tween 20. Nonspecific antibody binding was prevented by treating cells with a blocking solution of PBS-T 1% BSA. Primary antibodies (Table 2) diluted in blocking solution were applied either for one hour at room temperature or overnight at 4^{o}C. Primary antibodies were incubated for 24 hours

before being washed out with PBS-T. Secondary antibodies (Table 3) diluted in blocking solution were applied for one hour at room temperature or 4^{o}C overnight. The secondary antibodies were washed out with PBS-T after incubation and a 1:1000 dilution of a 10 mg/ml stock solution of DAPI was applied.

Stained cells were imaged and then counted using Image-J software. The number of cells successfully infected with the Ad-PNMa and Ad-PN vectors was determined by taking the proportion of Pdx1-positive cells to DAPI-positive cells. The number of cells successfully infected with the Ad-PNMb vector was measured by taking the proportion of MafB-positive cells to DAPI-positive cells.

2.4 | RNA Extraction from HepG2 cells

RNA was extracted from HepG2 cells at the end of the 72-hour incubation period following Ad-PNMa infection. The procedure followed that provided by the Qiagen RNeasy Plus Mini Kit. RNA concentrations were measured using a Thermo Scientific NanoDrop Lite Spectrophotometer.

2.5 | cDNA Synthesis

cDNA synthesis was performed using the Life Technologies SuperScript III First-Strand Synthesis System. Polymerization cycles were performed in a Bio-Rad T100 Thermal Cycler.

2.6 | qRT-PCR for Insulin Gene Expression

qRT-PCR was performed to quantify the amount of insulin gene expression in HepG2 cells. Human Gapdh and insulin gene primers (Table 4) were PrimeTime probes from Integrated DNA Technologies. PCR cycles consisted of two minutes at 50^{o}C and five minutes at 95^{o}C,

followed by 40 cycles of 15 seconds at 95°C, one minute at 59°C, and 15 seconds at 72°C. Cycles were performed using an Eppendorf Mastercycler RealPlex^2. All experimental and control treatments were tested for insulin and Gapdh gene expression. A negative control of sterile water was used to ensure there was no sample contamination.

2.7 | Calculation of Fold Insulin Gene Expression Change

Fold insulin gene expression change was calculated to determine the effect of Ad-PNMa infection of HepG2 cells on insulin gene expression compared to untreated cells. For all Ad-PNMa treatments and the negative infection control, the ΔCt value for a single treatment was calculated as

$$\Delta Ct = \frac{Insulin\ Gene\ Expression}{Gapdh\ Gene\ Expression}.$$

The ΔΔCt value for one treatment was then calculated as

$$\Delta\Delta Ct = (\Delta Ct\ Experimental) - (\Delta Ct\ Control).$$

The fold insulin gene expression change for an Ad-PNMa treatment compared to the negative infection control was finally calculated as

$$Fold\ Expression\ Change = 2^{-\Delta\Delta Ct}.$$

3 | Results

3.1 | HepG2 A12 Ad-PNMa Assay

The mouse Ad-PNMa vector has been previously shown to be able to reprogram mouse liver cells *in vivo* (Banga et al., 2012, 2014). The human HepG2 hepatocyte cell line was determined to be a suitable cell line studies with human cells (Knowles and Aden, 1983). The

vector is known to be able to infect multiple rodent cell types (Akinci et al., 2013; Banga et al., 2012, 2014), but human cell lines have not been extensively tested. HepG2 cells were infected with the A12 Ad-PNMa vector and incubated for either three or five days after infection. The cells were then fixed and tested for insulin and vector cargo protein expression. Cells not infected with the Ad-PNMa vector were used as a negative control. Positive Pdx1 expression was seen in HepG2 cells infected with Ad-PNMa. Weak insulin protein expression was detected in both negative control and five-day infected cells (Figure 5), so it was determined that insulin protein expression could not be reliably detected after this treatment using antibody detection. The three-day infection resulted in lower numbers of infected cells expressing Pdx1 protein than did the five-day infection (Figure 4), so the five-day infection was selected for the remaining HepG2 experiments. The five-day infection produced a maximum of 71% of infected cells expressing Pdx1 protein when treated with $7.88x10^9$ ifu/ml of A12 Ad-PNM, demonstrating that HepG2 cells can be efficiently infected and that greater vector volumes produced higher levels of Pdx1 expression.

3.2 | qRT-PCR of A12 Ad-PNM Infected HepG2 Cells

To determine whether A12 Ad-PNM was inducing insulin gene expression in HepG2 cells, qRT-PCR was used to quantify the level of gene expression. Only the five-day vector infection was used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene. A12 Ad-PNM treatment was determined to induce insulin gene expression in HepG2 cells (Figure 6), whereas the negative control without vector addition did not induce insulin gene expression. Increasing the volume of vector did not significantly affect the expression of insulin protein.

3.3 | HPDE C13 Ad-PNM Assay

The capacity of HPDE cells to be reprogrammed using C13 Ad-PNM was performed to provide a comparison to the HepG2 cell line. HPDE cells were selected for subsequent reprogramming experiments because they are a stable human pancreatic ductal cell line (Furukawa et al., 1996; Ouyang et al., 2000), which have been previously reprogrammed into β-cells using similar methods (Lee et al., 2013a). To assess infection of HPDE cells, mouse C13 Ad-PNMa vector was applied and cells were incubated for three days prior to antibody detection for insulin and vector proteins. Positive expression of Pdx1 protein was detected in HPDE cells infected with C13 Ad-PNMa, however there was no insulin expression detected in the cells (Figure 7). Additionally, higher volumes of the vector corresponded to greater numbers of HPDE cells expressing Pdx1, up to 85% of total cells at a concentration of $1.52x10^{10}$ ifu/ml (Figure 8). Treatment of infected cells with an anti-MafB antibody detected protein expression, despite this vector not containing a MafB component (Figure 9). This was determined to be due to cross-detection with MafA protein.

3.4 | HPDE Ad-PN Assay

The Ad-PN vector was assayed as a preliminary indicator of whether Pdx1 and Ngn3 would be sufficient to induce insulin expression in HPDE cells. Although both MafA and MafB are required for expression of insulin during development (Artner et al., 2007, 2008; Conrad et al., 2015; Dai et al., 2012), it is possible expressing Pdx1 and Ngn3 would be enough to induce reprogramming. Determination of Ad-PN infection efficiency was performed by applying the vector to HPDE cells and incubating them for three additional days. Antibody detection was then used to identify expression of insulin and vector proteins. Weak expression of Pdx1 was shown in treated HPDE cells, though no insulin was detected (Figure 10). The percent of Pdx1-positive

cells was very low, with the maximum being 7.3% at a vector dilution of $2.00x10^9$ ifu/ml, and increasing the vector concentration did not affect expression level (Figure 11).

3.5 | HPDE Ad-PNMb Assay

Infection with the Ad-PNMb vector was performed to assess whether the human three-gene combination of Pdx1, Ngn3, and MafB could induce insulin expression in HPDE cells. MafB is shown to have a different developmental role in human β-cells than in mouse β-cells (Conrad et al., 2015; Dai et al., 2012). HPDE cells were infected with Ad-PNMb and incubated for three days prior to antibody detection of insulin and vector proteins. It was shown that positive expression of MafB protein could be induced in the HPDE cells, but antibody detection could not readily detect insulin (Figure 12). Higher volumes of vector resulted in a greater number of cells expressing MafB, up to 64% of total cells at $1.70x10^{10}$ ifu/ml of vector (Figure 13). This indicates the Ad-PNMb vector can successfully infect the HPDE cells, but is less effective at infecting HPDE cells than the C13 Ad-PNMa vector.

4 | Discussion

This study investigated the potential to reprogram human cell lines towards a β-cell-like phenotype whereby the reprogrammed cells secrete insulin and may be used in future clinical studies for the treatment of type 1 diabetes. Reprogramming experiments such as this have become increasingly important for evaluating potential type 1 diabetes treatments, as the ability to create an endless bank of insulin producing cells for transplantation combats the significant issue of insufficient donor β-cells, islets, or whole pancreases (Landsberg and Shapiro, 2010). Efforts to reprogram cells to express insulin in mice have been successful in the past using three transcription factors important to β-cell development and maturation, Pdx1, MafA, and Ngn3

(Banga et al., 2012, 2014; Zhou et al., 2008). In this work, the cell reprogramming using Pdx1, Ngn3, and MafA, and the additional transcription factor MafB was also introduced in place of MafA because of the more prominent role MafB has in supporting insulin secretion in mature human β-cells as opposed to mouse cells (Conrad et al., 2015; Dai et al., 2012).

Two human cell lines were infected and tested for both vector and insulin gene expression to assess the viability of using these gene combinations for reprogramming. It has been demonstrated that infection with the Ad-PNMa vector is capable of inducing insulin gene expression in the human liver HepG2 cell line, but that insulin expression was not detected by immunohistochemistry in the HPDE cell line after treatment with the Ad-PNMa, Ad-PN, or Ad-PNMb vectors. The induction of insulin gene expression detected in HepG2 cells following Ad-PNMa indicates that exogenous co-expression of the three-genes Pdx1, Ngn3, and MafA can induce a change in endogenous gene expression in the HepG2 cells. Although the Ad-PNMa, Ad-PN, and Ad-PNMb vectors were shown capable of infecting the HPDE cells, the experiments reported here indicate these combinations of genes are not sufficient to induce reprogramming in this cell line.

The HepG2 cells were shown capable of being partially reprogrammed following Ad-PNM treatment, but the HPDE cells were not, indicating there may be some inherent property of the HepG2 phenotype that makes their reprogramming to a pancreatic phenotype easier. It might be expected that the pancreatic-origin HPDE cells could cause them to be more readily reprogrammed to the phenotype of a different pancreatic cell than a liver cell line. Liver cells have previously been shown to be excellent targets of reprogramming therapies (Banga et al., 2012, 2014; Li et al., 2005; Yang et al., 2010), and their close developmental origin to the pancreas has been suggested as one reason for this (Reviewed in Slack, 1995). It does remain

possible that the HPDE cells were able to express some amount of insulin in response to the vectors, but the expression was too low to be detected by immunostaining. qRT-PCR analysis of gene expression in Ad-PNM or Ad-PN vector treated HPDE cells would provide the most conclusive evidence of whether insulin RNA was being expressed.

Expression of the Ad-PN vector Pdx1 gene in HPDE cells was much weaker than expression of Pdx1 in Ad-PNMa HepG2 and HPDE treated cells or MafB in Ad-PNMb treated HPDE cells. This and the observation of a greater abundance of HPDE cells after infection with Ad-PN compared to after infection with either Ad-PNMa or Ad-PNMb suggests that the Ad-PN vector is unable to infect the HPDE cells with high efficiency. It is unknown why this vector is significantly less effective than Ad-PNMa or Ad-PNMb, though it is possible that higher vector concentrations at the time of infection would show greater infection efficiency.

Although the qRT-PCR performed on the treated HepG2 cells showed insulin expression at nearly $5x10^3$ fold greater than the negative control that showed nearly undetectable expression, the absolute level of induced insulin gene expression is still very low. The relatively weak expression of insulin in treated HepG2 cells and lack of expression in Ad-PNMa-treated HPDE cells may be due to the ineffectiveness in the expression of exogenous mouse proteins from the Ad-PNMa vector to reprogram human cells. As discussed previously, MafA is solely responsible for inducing insulin expression in the adult mouse β-cell (Artner et al., 2010; Nishimura et al., 2006), whereas the human β-cell expresses both MafA and MafB expression (Conrad et al., 2015; Dai et al., 2012). Expressing only MafA in human cells may eliminate induction of transcription factor targets in the reprogrammed cells that are necessary for insulin expression in mature human β-cells. In addition, mouse and human Pdx1 proteins largely induce species-specific expression of gene-targets, though there is some ability for mouse Pdx1 to induce human

genes and vice versa (Benner et al., 2014). Taking this into account, the results reported here for reprogramming human cells using vectors expressing mouse coding sequences may be consistent with the differential transcriptomes seen in mouse and human β-cells.

Inducing significant insulin protein expression in human cells may also require additional transcription factors, or other methods to enhance the reprogramming process. Other genes to include in a reprogramming should be capable of inducing a large number of human-specific β-cell genes. The inclusion of different genes can be in addition to the three already in use, or as a replacement to one or more of them. The six genes NeuroD, Nkx2.2, Nkx6.1, Pax4, Pax6, and Isl1 have been previously indicated as having potential in reprogramming towards a β-cell phenotype (Zhou et al., 2008). Alternatively, small molecules and other chemicals could be applied to cells in the hopes of enhancing the efficacy of reprogramming treatments. One such chemical is Polyinosinic-polycytidylic acid, an analog of dsRNA that triggers toll-like receptors to induce a cellular innate immune response (Lee et al., 2013b). The activation of the innate immune system has been shown to enhance reprogramming due to the induction of epigenetic chromatin modifications (Lee et al., 2013b; O'Neill, 2012). Finally, as seen in the difference in percent infected HepG2 cells treated with Ad-PNMa between three and five days, it is possible longer treatment and cell incubation times may lead to a greater proportion of reprogrammed and insulin gene expressing cells.

In conclusion, the study reported here indicates that treatment with Ad-PNMa can weakly induce insulin gene expression in HepG2 cells, but that no insulin protein expression can be conclusively detected in HPDE cells in response to exogenous expression of mouse Ad-PNMa or human Ad-PNMb and Ad-PN genes. Insulin expression was only reliably detected using qRT-PCR. The lack of insulin expression seen in the HPDE cells is not conclusive, and it is

recommended that more trials be performed using qRT-PCR analysis to quantitate expression. Due to the large difference between mouse and human gene expression landscapes, it is important to recognize that the mouse studies that were the basis for these experiments may need to be significantly modified in order to achieve success. Future studies using alternative combinations of transcription factors in the vector may provide the best path towards inducing positive insulin protein expression in human cells.

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Tables

Table 1. Titers for the Ad-PNMa, Ad-PN, and Ad-PNMb vectors.

Virus	Titer (ifu/ml)
A12 Ad-PNM	$1.97x10^{10}$
C13 Ad-PNM	$7.6x10^{10}$
Ad-hPNMb	$8.5x10^{10}$
Ad-hPN	$1.0x10^{10}$

Table 2. Primary antibodies and dilutions.

Primary Antibody	Dilution
Millipore Anti-Pdx1 Rabbit	1:2000
Millipore Anti-MafB Rabbit	1:1000
Cell Marque Anti-Insulin Guinea Pig	1:500

Table 3. Secondary antibodies and dilutions.

Secondary Antibody	Dilution
Alexa Fluor 488 Goat Anti-Rabbit	1:1000
Alexa Fluor 555 Donkey Anti-Guinea Pig	1:500

Table 4. PrimeTime primers for human Gapdh and insulin genes used for qRT-PCR. A 5' fluorophore and 3' quencher is attached to the probe. An internal quencher is designated by the break in probe sequence.

Target Gene	Primer	Sequence
Gapdh	Forward	TGTAGTTGAGGTCAATGAAGGG
	Reverse	ACATGCTCAGACACCATG
	Probe	AAGGTCGGA//GTCAACGGATTTGGTC
Insulin	Forward	CTTCACGAGCCCAGCCA
	Reverse	ATCAGAAGAGGCCATCAAGC
	Probe	CCTGAGCCC//ACCTGACGCAAAG

Figures

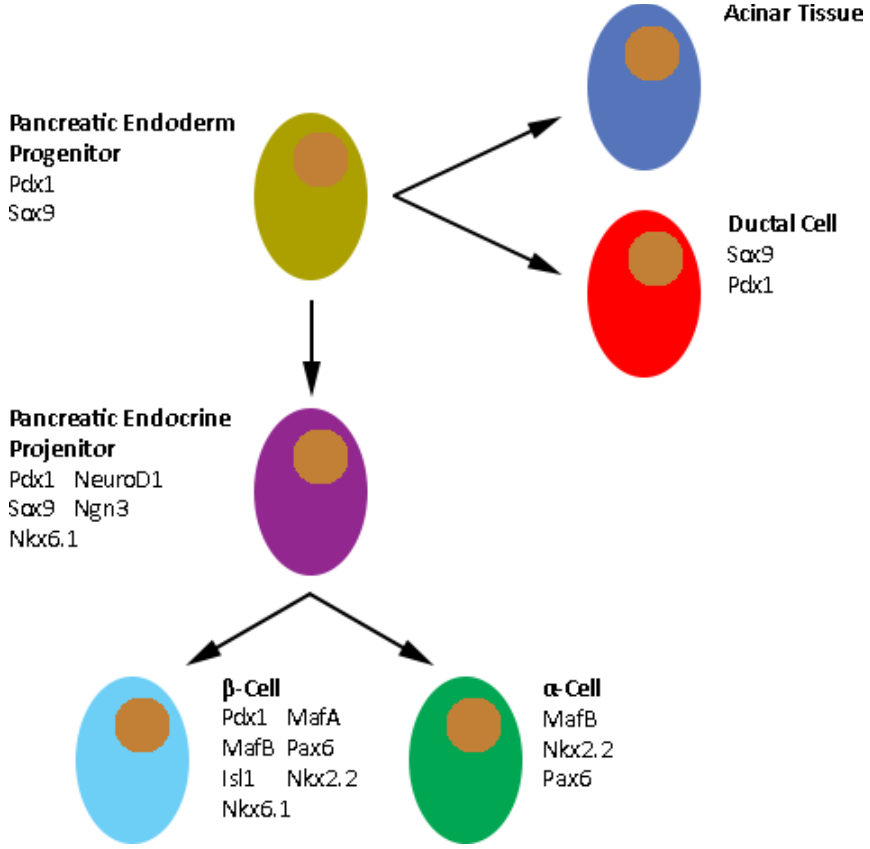

Figure 1. Transcription factors involved in pancreatic tissue specification. Development order from (Jennings et al., 2013, 2015; Slack, 1995).

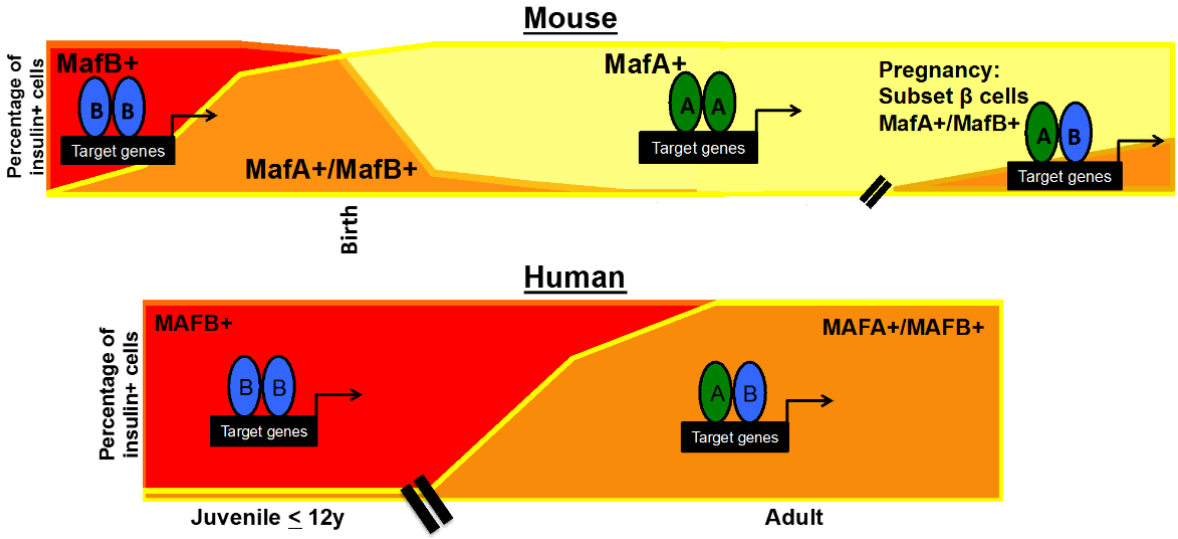

Figure 2. Differential expression over time of MafA and MafB transcription factors in the adult β-cells of humans and mice (Roland Stein, Vanderbilt University, personal communication).

Figure 3. Format of the Ad-PNMa vector. Adapted from (Banga et al., 2012).

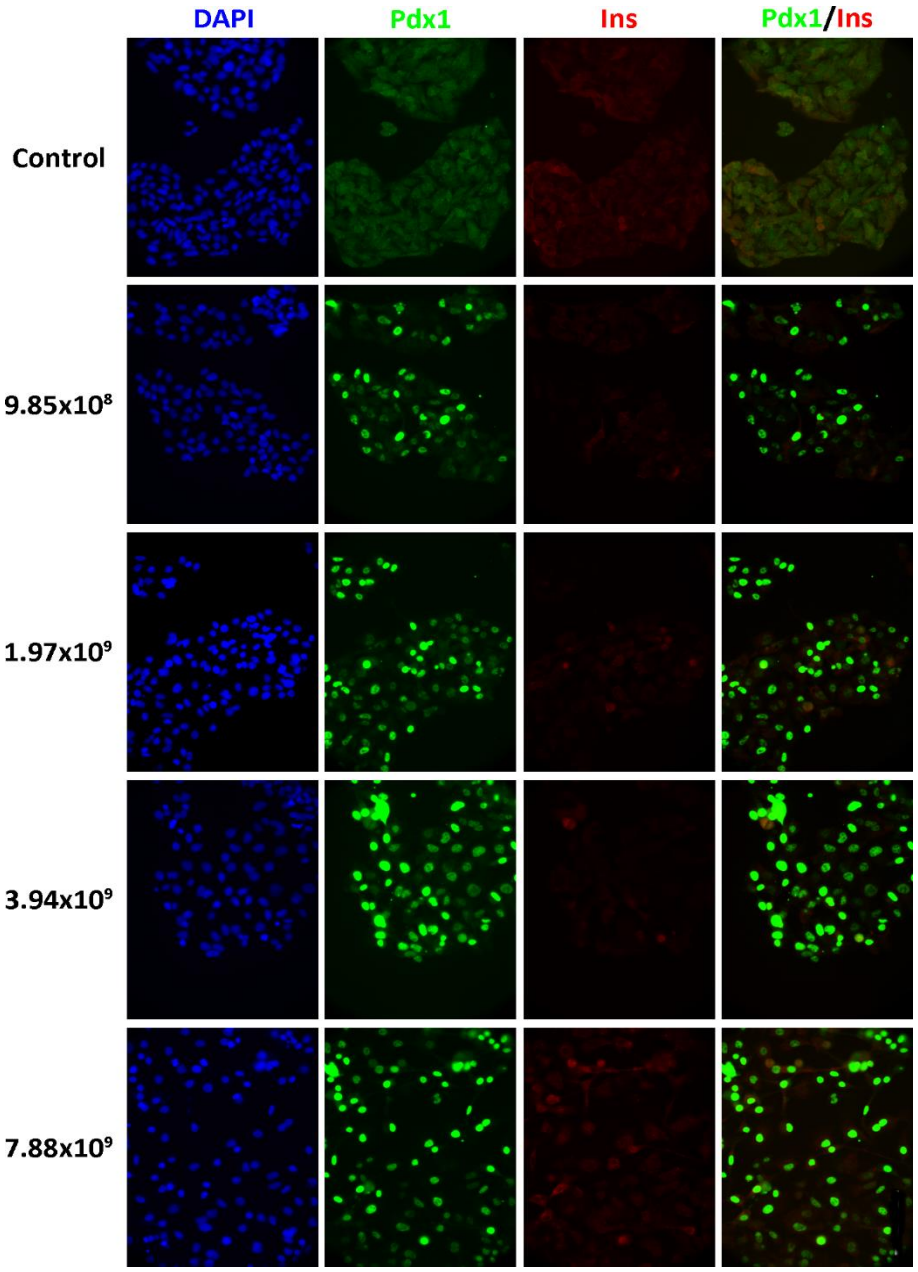

Figure 4. Antibody detection of Pdx1 and insulin in HepG2 cells treated with dilutions (ifu/ml) of A12 Ad-PNM. Vector titer is reported as $1.97x10^{10}$ ifu/ml prior to dilution 1:1000. Cells were infected with vector for 120 hours prior to fixing and being tested with antibody detection for Pdx1 and insulin. Wells of uninfected HepG2 cells were used as a negative control. Cells expressing positive Pdx1 show a nuclear concentration of the protein, whereas positive insulin shows a cytoplasmic concentration of protein.

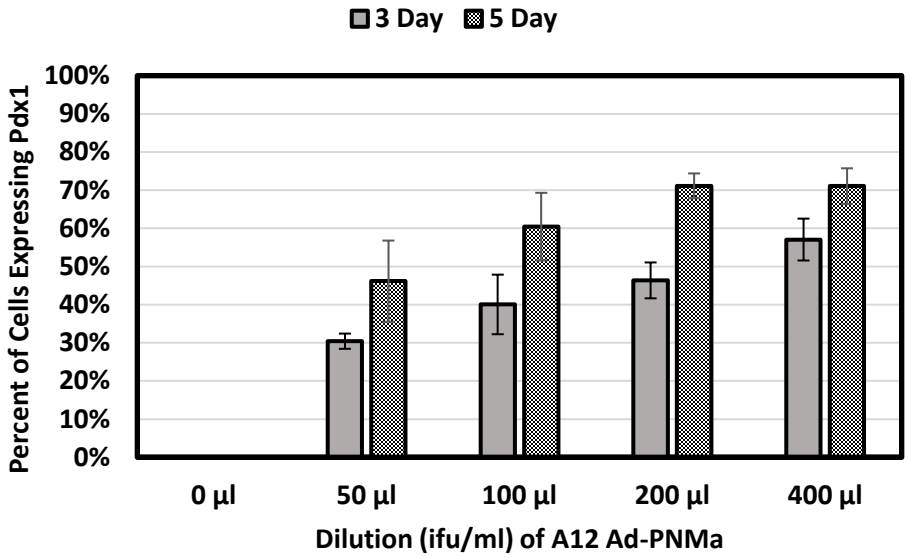

Figure 5. Percent expression of Pdx1 protein in HepG2 cells infected with dilutions of A12 Ad-PNM. Vector titer is $1.97x10^{10}$ ifu/ml prior to dilution 1:1000. Twelve-well plates of 50,000 cells/well were used. Cells were infected with vector for either 72 or 120 hours. The 72-hour infection was incubated for an additional 48 hours after infection. Uninfected HepG2 cells were used as negative controls. Quantification was performed by counting Pdx1-positive cells as a proportion of DAPI-positive cells. Results are reported as the mean percent of Pdx1-positive cells for three independent trials.

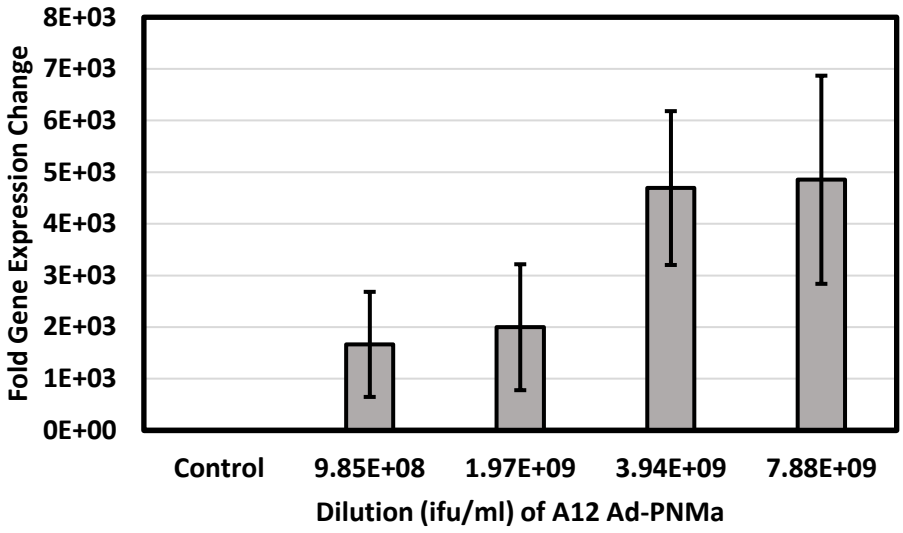

Figure 6. Fold expression change of insulin gene expression in HepG2 cells infected with dilutions of A12 Ad-PNMa. RNA was extracted and cDNA synthesized 120 hours after initial Ad-PNMa infection. Six-well plates of 100,000 cells/ well were used. GAPDH was used as the reference gene to calculate Ct values. Results are reported as the average fold change of three independent trials. Error bars represent one standard deviation.

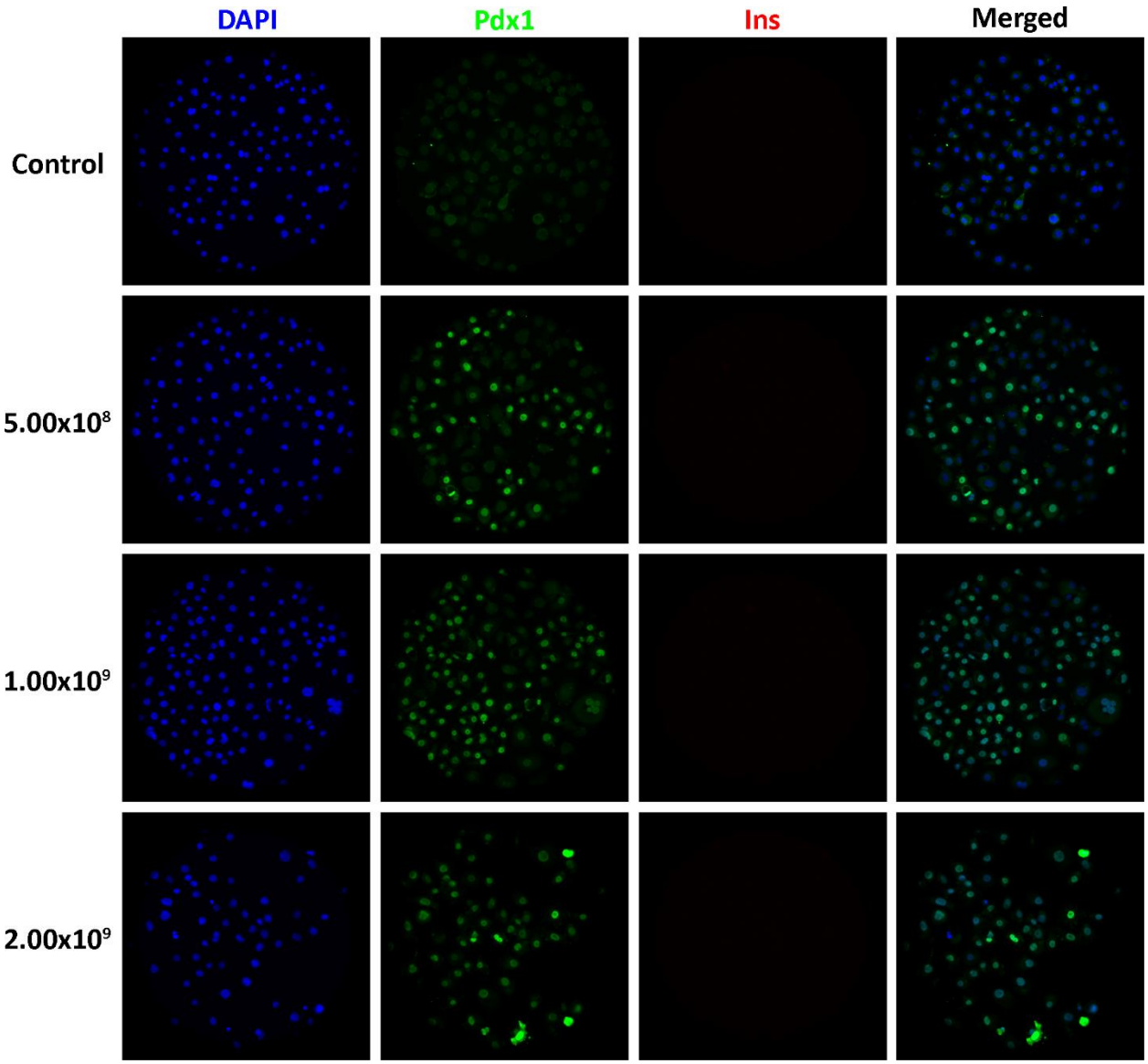

Figure 7. Antibody detection of Pdx1 and insulin in HPDE cells treated with dilutions (ifu/ml) C13 Ad-PNM. Vector titer is reported as $7.6x10^{10}$ ifu/ml prior to dilution 1:1000. Twelve-well plates of 50,000 cells/well were used. Cells were infected with vector for 24 hours and incubated for an additional 72 hours prior to antibody detection. Uninfected HPDE cells were used as a negative control. Cells expressing positive Pdx1 show a nuclear concentration of protein.

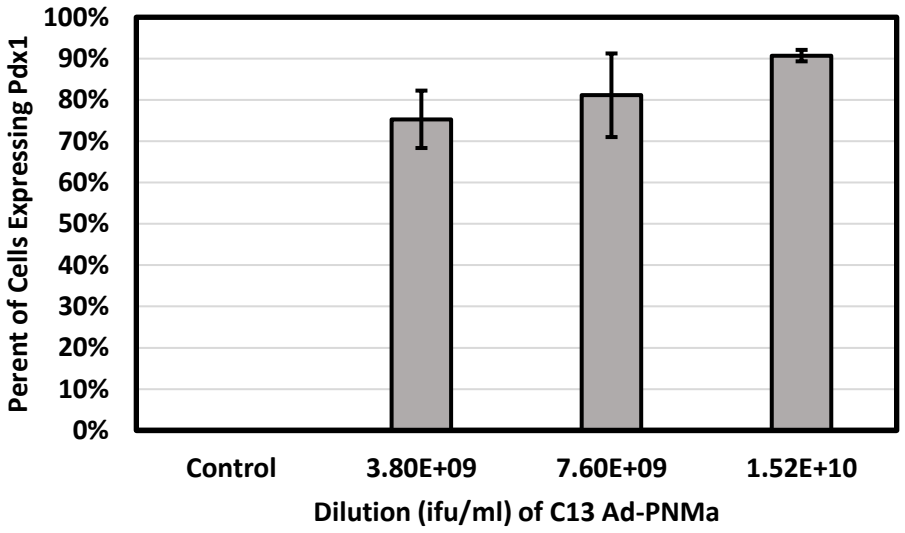

Figure 8. Percent expression of Pdx1 protein in HPDE cells transfected with dilutions of C13 Ad-PNMa. Vector titer is reported as $7.6x10^{10}$ ifu/ml prior to dilution 1:1000. Twelve-well plates of 50,000 cells/well were used. Cells were infected with vector for 24 hours and incubated for an additional 72 hours prior to antibody detection. Uninfected HPDE cells were used as a negative control. Quantification was performed by counting Pdx1-positive cells over DAPI-positive cells. Results are reported as the mean percent of Pdx1-positive cells for 3 independent trials. Error bars represent one standard deviation.

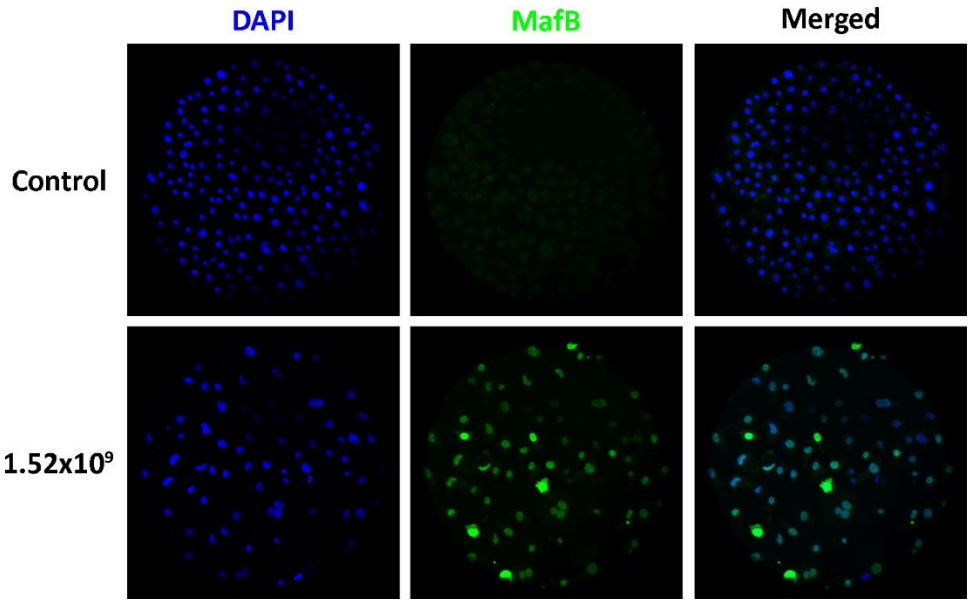

Figure 9. Antibody detection of MafB in HPDE cells treated with a dilution (ifu/ml) of Ad-PNMb. Vector titer is reported as $7.6x10^{10}$ ifu/ml prior to dilution 1:1000. Twelve-well plates of 50,000 cells/well were used. Cells were infected with vector for 24 hours and incubated for an additional 72 hours prior to antibody detection. Uninfected HPDE cells were used as a negative control. Cells positive for MafB show a nuclear protein concentration.

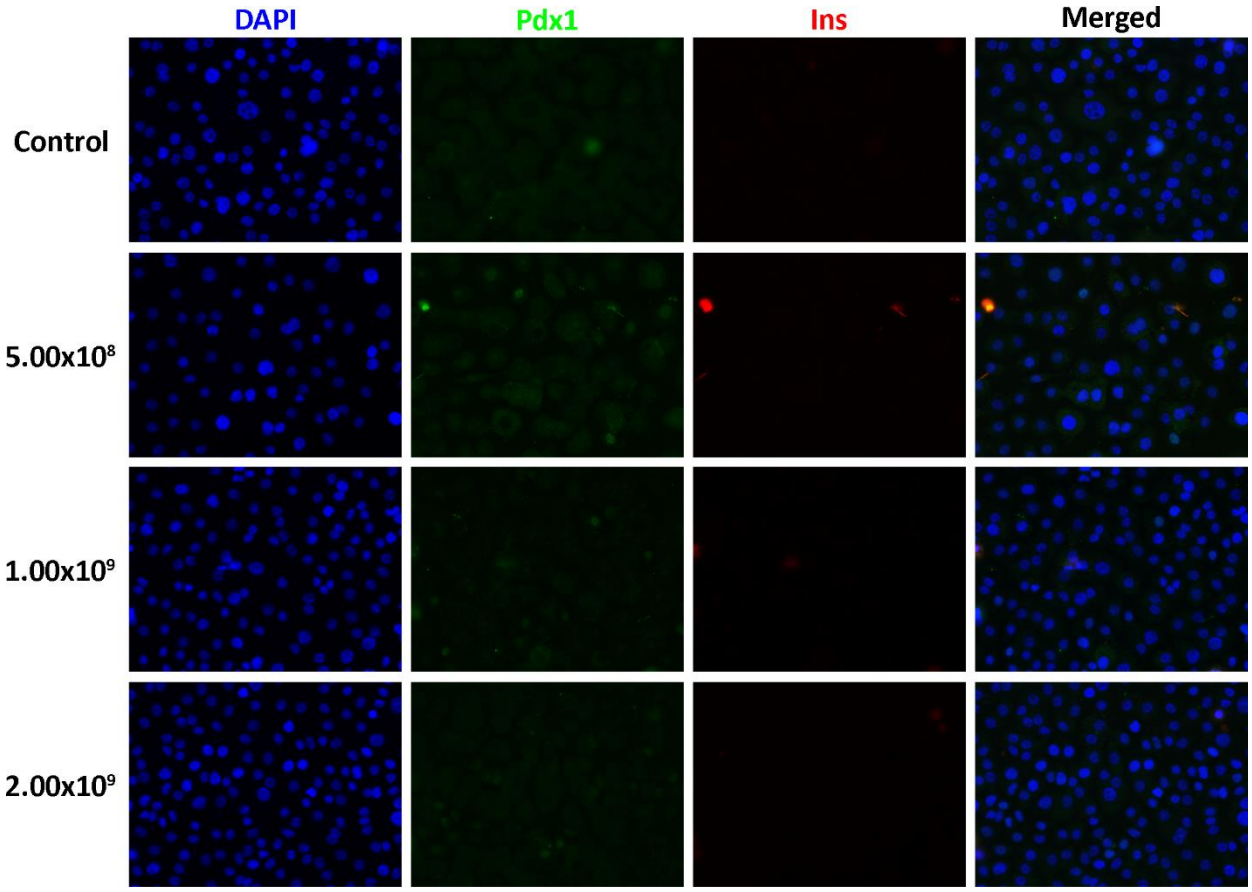

Figure 10. Antibody detection of Pdx1 and insulin in HPDE cells treated with dilutions (ifu/ml) Ad-PN. Vector titer is reported as 1.00x10^{10} ifu/ml prior to dilution 1:1000. Twelve-well plates of 50,000 cells/well were used. Cells were infected with vector for 24 hours and incubated for an additional 72 hours prior to antibody detection. Uninfected HPDE cells were used as a negative control. Cells expressing positive Pdx1 show a nuclear concentration of protein.

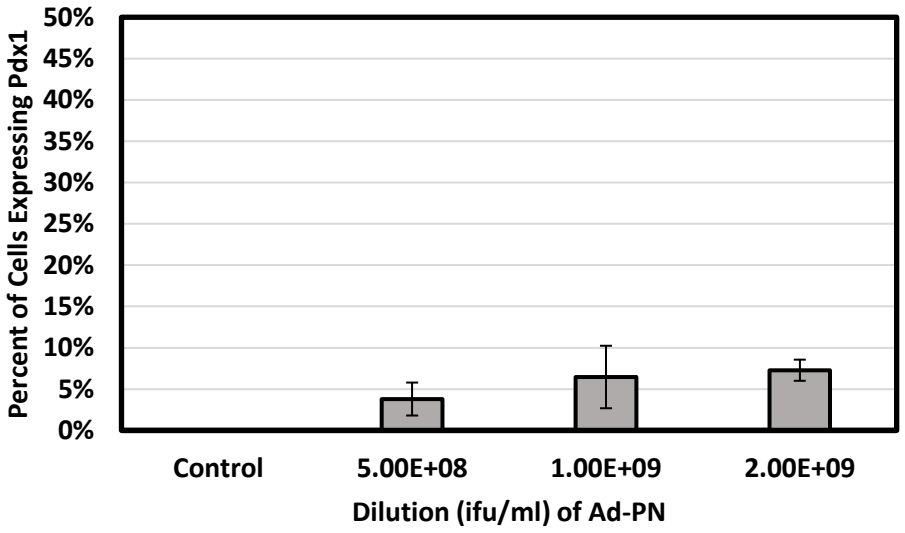

Figure 11. Percent expression of Pdx1 protein in HPDE cells transfected with dilutions of Ad-PN. Vector titer is reported as $1.00x10^{10}$ ifu/ml prior to dilution 1:1000. Twelve-well plates of 50,000 cells/well were used. Cells were infected with vector for 24 hours and incubated for an additional 72 hours prior to antibody detection. Uninfected HPDE cells were used as a negative control. Quantification was performed by counting Pdx1-positive cells over DAPI-positive cells. Results are reported as the mean percent of Pdx1-positive cells for 3 independent trials. Error bars represent one standard deviation.

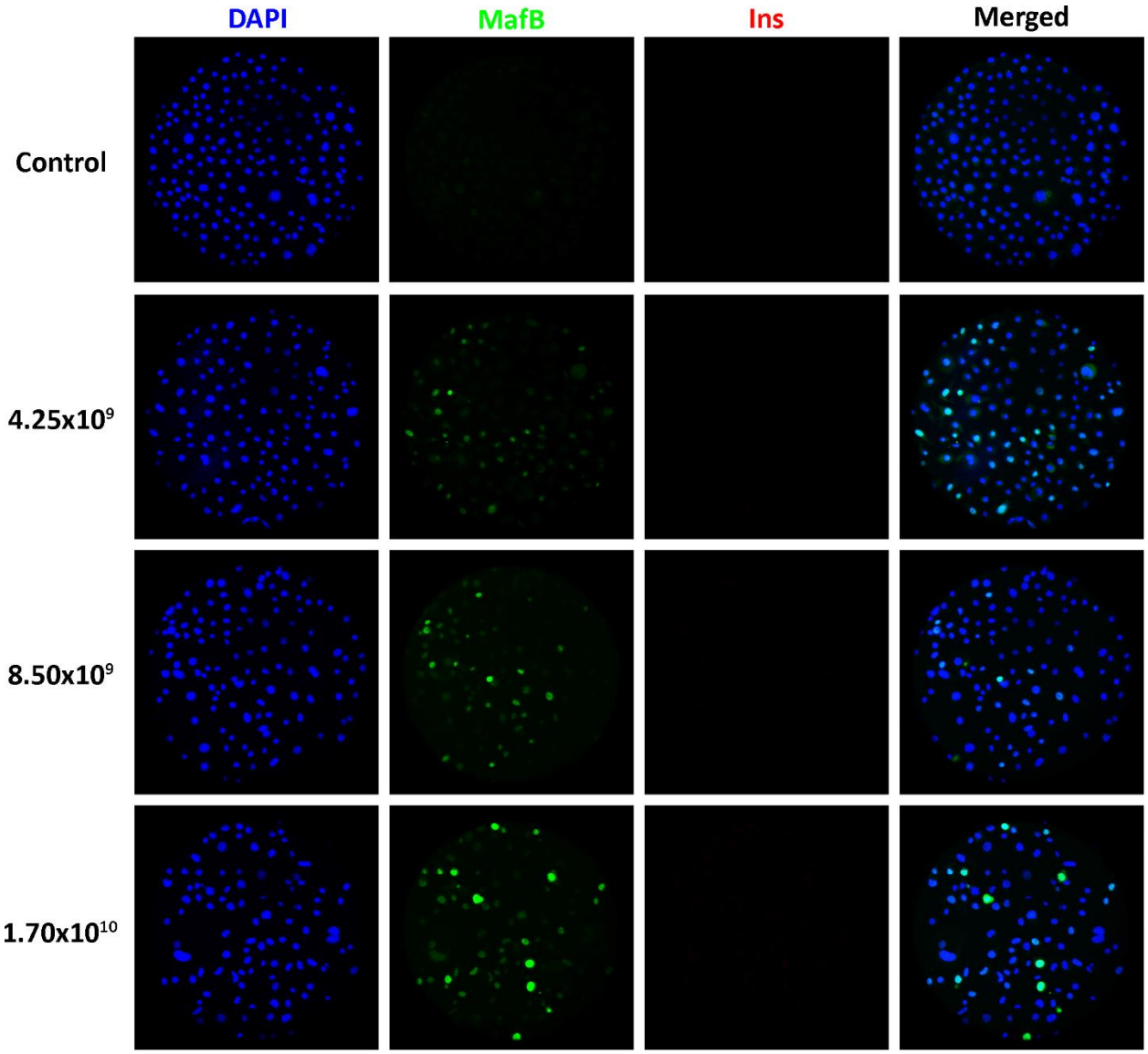

Figure 12. Antibody detection of MafB and insulin in HPDE cells treated with dilutions (ifu/ml) of Ad-PNMb. Vector titer is reported as 8.5x10^{10} ifu/ml prior to dilution 1:1000. Twelve-well plates of 50,000 cells/well were used. Cells were infected with vector for 24 hours and incubated for an additional 72 hours prior to antibody detection. Uninfected HPDE cells were used as a negative control. Cells expressing positive MafB show a nuclear concentration of the protein.

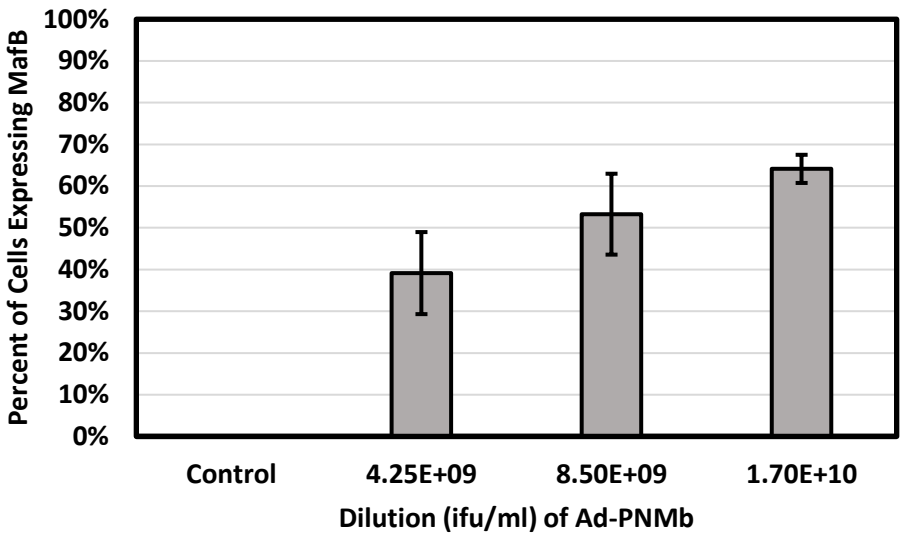

Figure 13. Percent expression of MafB protein in HPDE cells transfected with dilutions of Ad-PNMb. Vector titer is reported as $8.5x10^{10}$ ifu/ml prior to dilution 1:1000. Twelve-well plates of 50,000 cells/well were used. Cells were infected with vector for 24 hours and incubated for an additional 72 hours prior to antibody detection. Uninfected HPDE cells were used as a negative control. Quantification was performed by counting MafB-positive cells over DAPI-positive cells. Results are reported as the mean percent of MafB-positive cells for 3 independent trials. Error bars represent one standard deviation.